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F.J. Jimenez-Martín: Data curation, Formal analysis, Investigation, Methodology and Writing - original draft.

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**Title Page**

# Wing morphometrics for identification of forensically important blowflies (Diptera: Calliphoridae) in Iberian Peninsula

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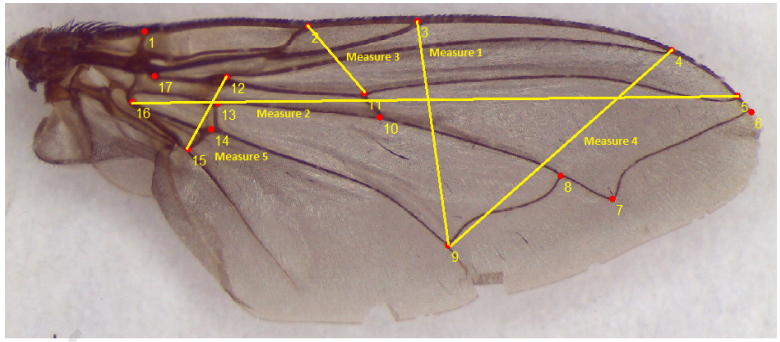
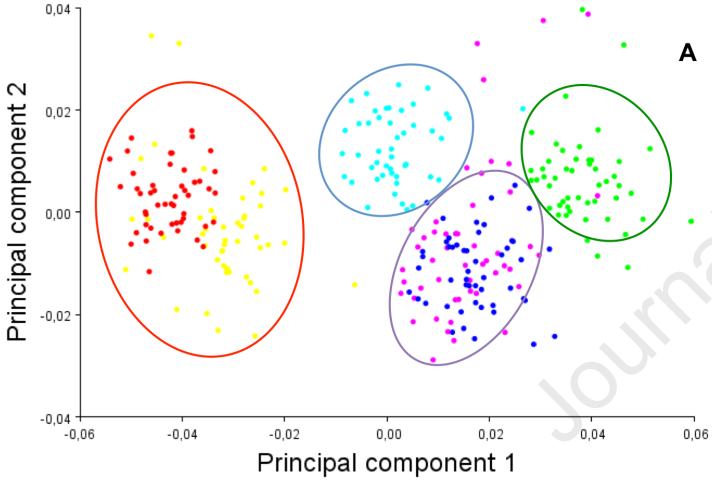
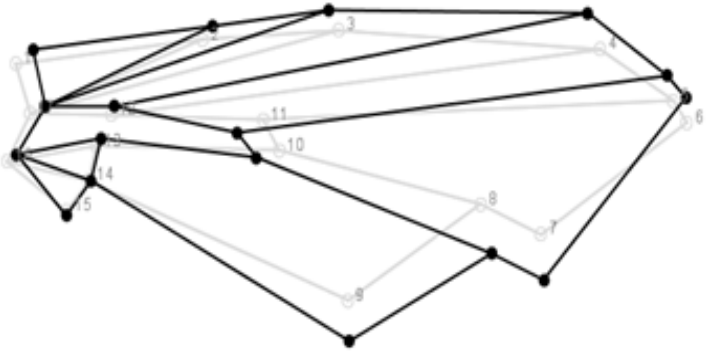
# Wing morphometric: Classical vs. Geometric

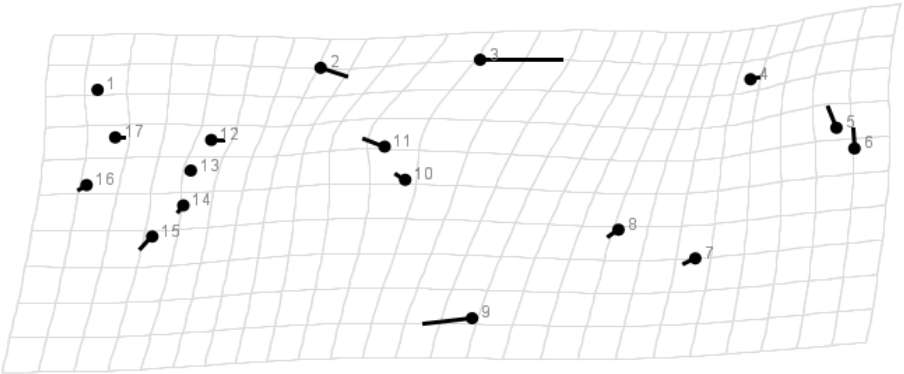



*Landmarks and* measures used in geometric and classical morphometrics

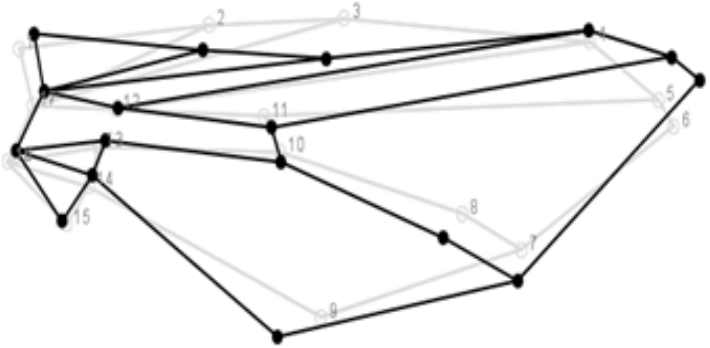



Principal component analysis representation (shape) to distinguish species






Deformation grid showing differences from average conformation



Consensus or “model” wings of the studied species

# Wing morphometrics for identification of forensically important blowflies (Diptera: Calliphoridae) in the Iberian Peninsula


## ABSTRACT

Calliphoridae is a family of Diptera of great forensic interest because some of its species belong to the sarcosaprophagous diptera community, as their larvae are necrophagous or necrophilous. In the Iberian Peninsula there are six species that stand out, as they are the first insects to arrive at carrion: *Calliphora vicina, Calliphora vomitoria, Lucilia sericata, Lucilia caesar*, *Chrysomya albiceps* and *Chrysomya megacephala.* To differentiate among these blowflies, we compared the value of using traditional and geometric morphometrics by performing a morphometric analysis of the wings (shape and size). A total of 600 individuals were considered, segregated by species and sex equally. Seventeen *landmarks* per wing were recognized, and then centroid size and shape analyses were performed using geometric morphometrics, and size using traditional morphometrics, to identify species and sex. The results showed differences in shape among the species and it was found that *landmarks* 2 to 11 were the most variable, while the landmarks located at the base of the wings were relatively stable. However, according to both traditional and geometric morphometrics, no significant differences were found among species in wing size (*Calliphora vicina*, *Chrysomya megacephala* and *Lucilia caesar* were indistinguishable), but females were larger than males (except for the two *Chrysomya* species). Our results indicate that the use of geometric morphometrics to analyze wing configuration (shape) is an easy-to-use tool that helps to distinguish among common blowfly species of forensic importance.


*Keywords:* Forensic entomology, Blowflies, Geometric morphometrics, Traditional morphometrics, Isometric size, Landmarks, Wing configuration, length measures.

## 1. Introduction

Necrophagous insects are those that feed on decaying organic matter of animal origin. They colonize corpses, and sometimes excrements, depositing their eggs, from which larvae will emerge and remain there up to the pupa stage. These insects are

attracted by gases produced by the body due to the degradation of organic compounds it contains [1]. For this reason, depending on the decomposition state of the corpse, certain species are attracted to the corpse in a clear succession, in groups called squadrons or legions [2]. These insects, mainly Diptera, are the most important because they arrive first, and their preimaginal stages allow the estimation of the post mortem interval (PMI), which is the time from the moment of death or colonization to the moment in which the corpse is discovered, in a broad sense [3-6]. Therefore, the identification of the species present at the crime scene is crucial in calculating the PMI. Taxonomic analysis, the size and the morphology of necrophagous insects, the estimation of PMI, and the determination of causes and place of death are the main topics studied in forensic entomology [4].

Calliphoridae and other Diptera families include species of forensic importance [7-8], so their taxonomy is very important to obtain information on the circumstances or the time of death. In Europe, the most common genera of Calliphoridae are *Lucilia* Cassini, 1817, *Calliphora* Robineau-Desvoidy, 1830, and *Chrysomya* Robineau-Desvoidy, 1830. In the Iberian Peninsula, the species related to human corpses are mainly *Calliphora vicina* Robineau-Desvoidy, 1839, *Calliphora vomitoria* (Linnaeus, 1758), *Chrysomya albiceps* (Wiedemann, 1819) and *Lucilia sericata* (Meigen, 1826), but also *Lucilia caesar* (Linnaeus, 1758). The oriental latrine fly *Chrysomya megacephala* (Fabricius, 1794) has recently been found in carrion, and because this species is common in human autopsies in other countries where it is established, it probably has great potential as a forensic indicator in the coastal regions of Southern Europe in the coming years [9].

Generally, a criminalist has little entomological training, which implies a certain disadvantage when it comes to establish the date of death, because recognizing insect species is crucial. The traditional method of blowfly identification relies on the use of morphological taxonomic keys and, normally traditional morphometrics methods [10-13]. This is a laborious process that also requires a good condition of the specimens. In some cases, moreover, the collected insects comprise broken and fragmented specimens, and therefore identification becomes more complicated. For this reason, it is essential to find a new tool that allows species to be identified quickly, easily and efficiently. In some forensic contexts, geometric morphometrics could be considered as a new tool to identify individuals, through the study of homologous conformation

structures in related species [14]. This tool is based on morphology and statistical analysis, studying the variation of shape and size in specific structures within and among species [15]. Geometric morphometrics (GM), described by Bookstein [16] as the quantitative study of variation in biological forms, appeared in the late 20th century. Wing has been widely used as a model for GM in Diptera, given some experimental advantages to other organs like, adult wing is essentially bidimensional, which facilitates the characterization and interpretation of its morphological variation. GM differ from Traditional Morphometrics (TM) in that the latter consider measures, while GM are based on point coordinates, called *landmarks*, represented on a Cartesian plane. Geometric morphometrics have the advantage of understanding the form of an object directly, as a cohesive whole, rather than indirectly through fragmentary measurements in traditional approaches [17-18]. As a disadvantage, although GM are objective, they require a not simple methodology and traditional methods may in some specific cases be quicker, more direct, and more effective to apply.

TM has been used to distinguish in base to size variations in adults in base to larval density, fertility rate or populations origin [19-21], but this technical has been substituted by GM in the last decades. GM have allowed differentiation between anthropic and wild species [22-25], laboratory populations [26], to distinguish altitude variations in populations [27], or populations from different geographic areas [28-30], and, of course, as a taxonomic tool to differentiate among species [31-32]. The utility of this tool has been demonstrated to discriminate between species that are difficult to distinguish from a taxonomical point of view, and it has also been useful in studies identifying intra-specific variability [33-34].

In recent years, this tool has been used successfully to distinguish Diptera species of veterinary and forensic importance [35-38]. In the case of using GM as a tool to automate identification, mainly in large samples or to distinguish between different families or genera, or to identify females, which are more difficult to identify than males, *landmarks* and the same methodology must be used when wing venation is similar [36-37]. A large number of Muscidae and Calliphoridae species have been studied using GM with the same methodology and landmarks, and the results allow us to distinguish among the species, although not always, and the sex is not identified. However, if the objective is to analyze intraspecific variability, such as the origin of a population, numerous *landmarks* increased the robustness of the analysis [38]. But it is

not always possible to compare species from different families, because wing venation can be very different, as in the case of *Hermetia illucens* Linnaeus, 1758 (Diptera, Stratiomyiidae) [39-40].

Our research focuses on the use of GM to study wing architecture variation, determining differences and changes in size and conformation, as a support for the taxonomy of species within the Calliphoridae family that are of forensic importance in Spain, and therefore in the Iberian Peninsula, but also to identify the gender. However, collecting large numbers of landmark points from wing images can also be a tough and intensive process, and even obtaining images and morphometric data and their applications is not always possible [38]. For this reason, we propose to compare traditional techniques (length measurements) with the isometric size (centroid) and vein configuration (shape) in the wings, both at inter- and intraspecific levels. The shape and size can act as possible taxonomic characters, but also as a means to distinguish between sexes if differences are found. Therefore, the application of GM as a tool will allow the identification of these blowflies in an effective way.

## 2. Material and methods

The specimens used in this study were collected from different habitats in localities in the province of Alicante (Spain): Benidorm (Benidorm Island: 38º30’8”N, 0º7’49”W), Alicante (Nueva Tabarca Island: 38º10’00”N, 0º28’00”W), Villena (Sierra del Collado: 38º36’14.89”N, 0º47’46.98”W), Agres (Foia Ampla: 38º45’34.7”N, 0º30’22.5”W) and Bocairent (Mas del Parral: 38º45’24.6”N, 0º32’26.1”W). These specimens were captured with wind-oriented traps (WOTs) baited with liver [34], between 1997 and 1998, and identified according to Peris and González-Mora [10-12]. All the material is deposited in the Entomological Collection of the University of Alicante (CEUA).

The right wings of 100 *C. vicina*, 100 *C. vomitoria*, 100 *Ch. albiceps*, 100 *Ch. megacephala*, 100 *L. caesar* and 100 *L. sericata*, 50 of each sex, were dislocated at the basal articulation and mounted on glass microscope slides. The wings were digitized and 17 anatomical reference points (*landmarks*) corresponding to Type I (according to Bookstein [41]) were selected at the intersections between different wing veins (Fig. 1),

based on previous publications and successful results [28, 42]. These points are homologous and easily identifiable, and have a relative consistency in all the species studied. The *landmarks* (lm) are located at the insertion of the humeral crossvein with the costa (lm1), at the insertion of the subcostal (sc) with the costa (lm2), at the end of $R_1$ (lm3), at the end of $R_{2+3}$ (lm4), at the end of $R_{4+5}$ (lm5), at the joint of M with the margin (lm6), at the bend of the vein M (lm7), at the joint of dm-cu with M (lm8), at the joint of dm-cu with $CuA_1$ (lm9), at the joint of r-m with M (lm10), at the joint of $R_{4+5}$ with r-m (lm11), at the intersection of $R_{2+3}$ with $R_{4+5}$ (lm12), at the intersection of M with bm-cu (lm13), at the joint of bm-cu with $CuA_1$ (lm14), at the joint of $A_1$+$CuA_2$ with $CuA_2$ (lm15), at the joint of $A_1$+$CuA_2$ with $CuA_1$ and M (lm16), and at the joint of $R_1$ with the stem-vein (lm17). The coordinate pairs of lm configuration were recorded with the programs TPSUtil and TPSDig [43]. To minimize measurement errors, all the wings were digitized three times by the same co-author.

Centroid size (the square root of the sum of the squared distances of all the *landmarks* of the wing from their centroid -center of gravity-) or isometric estimator of size was calculated using TpsRelw and TpsDig [43-44]. The Kruskal-Wallis non-parametric test and Student's t-test were used to identify significant differences between pairs of species by means of the R software (version 3.4.0) [45]. To observe differences using traditional morphometrics, significant distances between *landmarks* based on PCA analysis (Statistica software, version 7.0 [46]) were taken using the program ImageJ2 [47], Measure 1 being the distance between lm3 and lm9, Measure 2 between lm16 and lm5, Measure 3 between lm2 and lm11, Measure 4 between lm4 and lm9, and Measure 5 between lm15 and lm12 (Fig. 2). These measurements are not based on any specific previous research and may not have been used to perform morphometric analyses previously, but they do highlight reference points and linear distances used to describe the wing of the taxa considered, especially at a family or genus level.

For the wing shape analysis, the 17 *landmarks'* geometric coordinate configurations were stored in tps-files for MorphoJ 1.06d analysis [48]. Assuming that all the observed variability was exclusively due to wing shape, a Procrustes superimposition was carried out, taking into account alignment according to the main axes. Then a matrix with the conformation of variables (Partial Warps, Pw) was extracted. By applying Principal Component Analysis (PCA), the average species configuration was calculated, and therefore the 17 *landmarks* were transformed into a

grid for each species and sex. Through the PCA analysis the most representative *landmarks* and their variation level in each species and sex were established. A Procrustes ANOVA analyzed significant differences in wing shape. To achieve a better interpretation of the variation, the shapes with variations for each species and sex were obtained by means of a wireframe representation (schematic representations in which the *landmarks* of each configuration are joined by lines). Finally, consensus wings were obtained by overlaying the images, using tpsSuper 2.04 [49].

## Results

Three male specimens of *Ch. albiceps* and one male and one female of *L. sericata* were eliminated in the analysis because the *landmarks* were not discernible, or the wings were deformed.

### a) Size

Regarding the interspecific differences, significant differences were found in the wing centroid size when males and females were compared ($X^2$ = 445.19; $p < 0.0001$). For this reason, independent analyses were performed for each sex. Pairwise comparison of centroid sizes showed differences ($p<0.05$), being the highest in *C. vomitoria*, followed by *C. vicina – L. caesar – Ch. megacephala*, then *Ch. albiceps*, being the smallest in *L. sericata*, both in males and in females (Table 1). *Calliphora vicina*, *L. caesar*, and *Ch. megacephala* had similar centroid sizes. Differences in centroid size were found between males and females in *C. vicina, C. vomitoria, L. sericata*, and *L. caesar*, with females being bigger than males, while in *Ch. albiceps* and *Ch. megacephala* there were no significant differences (Table 1).

According to traditional morphometrics, the PCA analysis indicated that the five distance measurements considered were positively correlated, which could be observed in the correlation matrix. Through the PCA analysis, the five measures were reduced to the least number of orthogonal factors. The PCA analysis recognized a unique factor that aggregated, according to its eigenvalue, 4.70 measurements and explained 93.91% of the total variance. Considering the *factor loadings*, Measure 2 proved to be the most correlated with the obtained factor. Consequently, this distance was used to identify differences in species and sexes by the Kruskal-Wallis non-parametric test. On the basis

of Measure 2 (Table 2), significant differences were found among species ($X^2$ = 447.37; p < 0.0001), except in the relationships between *C. vicina – L. caesar* (p = 0.3394), *C. vicina – Ch. megacephala* (p = 0.3672), and males of *Ch. megacephala – L. caesar* (p = 0.2694) although the more confident measurement was used. The results indicated that *C. vomitoria* has the longest wings, followed by *C. vicina – L. caesar-Ch. megacephala*, then *Ch. albiceps*, with *L. sericata* having the shortest. Intraspecific analysis to determine whether there was sexual dimorphism in wing size confirmed that females were significantly bigger than males, except for the *Chrysomya* species, where the sexes were similar in size.

**b) Shape**

Figure 3 shows the graphic PCA for males and females, since sexual differences were found (F = 41.29; df= 180; p<0.001; PC1 = 49.81 % and PC2 = 10.85 %). When all the wing configurations where compared with each other, taking into account the first two main components, a high level of similarity was observed between the *Chrysomya* and *Lucilia* species, and a lower level of similarity in the case of the *Calliphora* species, for both sexes. However, the shape allowed us to identify all the blowflies regardless of sex (F = 140.90; df= 300; p<0.001; PC1 = 49.80 % and PC2 = 10.85 %), and also within males (F = 79.65; df= 150; p<0.001; PC1 = 49.12 % and PC2 = 8.94 %) and within females (F = 96,28; df= 150; p<0.001; PC1 = 52.87 % and PC2 = 10.46 %) (Table 3).

Wing shape variation was inferred as deformations of the seventeen points in a grid, according to the results obtained by the PCA (Fig. 4). The most significant variations occurred in landmarks 3 and 9, at the anterior and posterior edge of the wing, while the landmarks with the least displacement were 1 and 13, located at the base. In general, lm 3 moved horizontally to the right, while lm 9 moved horizontally to the left or wing base. It is worth noting that lm 5 and lm 6 moved vertically towards the wing top. However, these results vary according to the species (Fig. 5). In the *Calliphora* species the most variable landmarks were 2, 3, 7, 8 and 9, while the landmarks located at the base of the wings were relatively stable. In both species, lm 2 and 3 moved towards the wing base horizontally; lm 7, 8 and 9 moved towards the outer posterior edge of the wing, diagonally in the case of *C. vicina* and horizontally in *C. vomitoria*. Regarding the *Chrysomya* species, lm 4, 7, 8 and 9 were more variable than the others,

but there were great differences between the two species studied. In the case of *Ch. albiceps*, lm 4 to lm 9 presented the greatest displacement, following a diagonal towards the costal vein on the anterior wing edge (lm 4 to lm 6), or following a diagonal line toward the posterior edge of the wing (lm 7 to lm 9). In *Ch. megacephala*, lm 2 to lm 4, lm 7 and lm 9 were the most displaced, but lm 5 and 6 were fixed. Lm 2 to lm 4 moved towards the inner part of the costal or anterior margin diagonally, but lm 7 and 9 were displaced towards the external border of the wing. Lm 10 and 11 were practically fixed. Finally, in the *Lucilia* species there were great variations in landmarks 5 to 9. In *L. caesar*, the most important *landmarks* were lm 8 and lm 9, which were displaced horizontally towards the wing base. For *L. sericata*, lm 5 to lm 9 were the most important, with lm 5 and lm 6 displaced diagonally to the external part of the costal vein, and lm 7 to lm 9 moving towards the posterior basal edge of the wing diagonally. Finally, lm 1 and lm 15 showed a big displacement in *L. caesar*, while in *L. sericata* they were fixed.

When considering sexual dimorphism (Fig. 6), it was observed that in the females of *Ch. albiceps* the *landmarks* that showed the greatest displacement were lm 4 to lm 9, with the same displacement previously described for this species. However, for males, *landmarks* lm 2 to lm 11 underwent the greatest displacement. Lm 2 moved diagonally to the upper-right wing area and lm 3 was displaced horizontally. *Landmarks* lm 4 to lm 9 moved vertically, similarly to females, while *landmarks* lm 10 and lm 11 moved diagonally toward the lower-right wing area. Fixed *landmarks* were lm 14 and lm 15 in females and lm 12 and lm 13 in males. The females of *Ch. megacephala* presented *landmarks* lm 2, lm 3, lm 6, lm 10, lm 11 and lm 17 as the most representative. *Landmarks* lm 2 and lm 17 moved diagonally to the upper area of the wing base, lm 6 was displaced towards lm 7, while lm 10 and lm 11 were displaced horizontally in the opposite direction to the wing base, and finally, lm 3 moved horizontally towards the wing base. The most important l*andmarks* in males of the same species were lm 5 to lm 7, and lm 10 and lm 11. *Landmarks* lm 5 and lm 6 moved diagonally towards lm 4, *landmarks* lm 10 and lm 11 moved diagonally to the lower left area of the wing, while lm 7 moved diagonally to the lower right area of the wing. Fixed *landmarks* were lm 12 and lm 14 in females, and lm 12 to lm 15 in males. In females of *C. vicina*, the most important *landmarks* were lm 5 to lm 9. *Landmarks* lm 5 and lm 6 moved diagonally in the opposite direction to lm 7. Lm 7 was displaced vertically to the

lower area of the wing, while *landmarks* lm 8 and lm 9 moved diagonally to the lower right area of the wing. In males, the *landmarks* with the greatest displacement were lm 5 to lm 11. Lm 5 and lm 6 moved vertically to the upper area of the wing; lm 8, lm 10 and lm 11 were displaced diagonally to the lower-right wing area, while lm 7 and lm 9 moved diagonally towards the lower base edge of the wing. Fixed *landmarks* were lm 2 in females and lm 14 in males. Regarding females of *C. vomitoria*, lm 3 to lm 9 were the most varied. *Landmark* lm 3 moved horizontally, lm 4 to lm 6 moved diagonally towards the upper edge of the wing, and lm 7 to lm 9 moved diagonally towards the lower edge of the wing. In males, the most displaced *landmarks* were lm 3, and lm 5 to lm 9. Lm 5 and lm 6 moved diagonally towards the upper-right wing area, and lm 7 to lm 9 diagonally towards the lower wing area, as in females, while lm 3 moved diagonally to lm 11. Fixed landmarks were lm 14 in females, and lm 12 and lm 15 in males. Finally, in the case of the genus *Lucilia*, the females of *L. caesar* presented *landmarks* lm 4 to lm 11 and lm 1 as representative. *Landmark* lm 4 moved diagonally towards lm 3; lm 5, lm 6 and lm 1 moved vertically to the upper wing area, lm 7, lm 8, lm 10 and lm 11 were displaced diagonally to the lower right wing area, and finally, lm 9 moved diagonally to the lower left wing area. In males, *landmarks* lm 1, lm 5, lm 6, lm 8 and lm 10 came out as representative. The position and displacement of lm 1 and lm 9 were remarkable, as well as the displacement of lm 5 and lm 6 with respect to females. Fixed *landmark* was lm 13 in females, and lm 12 and lm 13 in males. In females of *L. sericata* the *landmarks* lm 5, lm 6, lm 8 and lm 9 were the most important, with lm 5 and lm 6 displaced diagonally towards the upper right edge of the wing, and lm 8 and lm 9 moved diagonally to the lower left wing area. In males, the most important *landmarks* were lm 5 to lm 9, with the same displacement as in females. Fixed *landmarks* were lm 1, lm 3, lm 14 and lm 15 in females, and lm 2 in males.

Consensus wings or “model wings” were obtained for each species and each sex, and for this purpose, the production of PCA variation graphs was integrated into a *wireframe* scheme in addition to a photo overlay, which provided an image model (Annex I).

**Discussion**

Geometric morphometrics are a relatively old technique that helps us analyze characters such as size or shape. The first person to make a deformation matrix was D'Arcy Thompson [50], but then, in the 1970s, it became more popular thanks to Bookstein. Now these techniques are used more frequently thanks to the technical improvements that have been made in recent years (computers with greater capacity, digital photography, etc.) and the field of GM has matured into a rich and cohesive discipline for the study of shape variation and covariation [51]. In our case GM and TM were applied to the wings of Calliphoridae of forensic interest in the Iberian Peninsula, *Ch. albiceps, Ch. megacephala, C. vicina, C. vomitoria, L. caesar* and *L. sericata*. An analysis of the results for the wing configuration showed that it can also be used to differentiate them, as well as to identify gender, indicating the existence of sexual dimorphism.

Generally, to carry out a correct taxonomic classification of any Diptera specimen, it is necessary to recognize certain characters correctly, such as the disposition of setae on the body surface or measures of structures and appendices [13, 52,53]. For this reason, it is very important to use any type of tool that helps in taxonomic identification, such as GM, taking into account different points that are homologous among different Diptera, in this case, the areas of union between wing veins. Unlike traditional morphometrics where multivariate analysis of several linear measures (trusses) across the body form have used to quantify shape, GM construct a truss network to capture both the venation pattern and overall wing shape. Newer geometric morphometric methods claim to better estimate shape because they analyze the geometry among the locations of all landmarks simultaneously rather than the linear distances between pairs of landmarks [54]. Different studies, such as that of Klingenberg & Zakland [55], affirm that GM are useful in the phenotypic analysis of shape variation. López-García *et al.* [56] confirm that wing GM are efficient at distinguishing piophilids genera, but they are also valid at lower hierarchical levels (species, dimorphic species, populations and sexes). Vasquez & Liria [35] suggest that wing geometric morphometrics are an important method to discriminate among certain species of Calliphoridae, demonstrating this with *Ch. albiceps* and *Ch. megacephala*. More recent studies are those of Grzywacz *et al.* [37], in which the authors identify different Muscidae specimens of medical-legal importance using GM, and that of Szpila *et al.* [36], in which they identify Calliphoridae species of forensic importance in

Europe. Our results were similar to those obtained by Szpila *et al.* [36], but these authors found no differences at the intraspecific level, nor did they discriminated between the sexes. Hall *et al.* [57], concluded that wings being compared must be processed in the same way, ideally slide mounted and without a mix of wings of males and females.

Based on the *landmarks* selected in the present study, the utility of GM as a tool for taxonomic analysis has been confirmed, as well as their use in discriminating between sexes in the *Calliphora* and *Lucilia* species, although not for the *Chrysomya* species. These differences between the two studies, regarding sexual dimorphism, may be caused by the selected *landmarks*. In both studies, the *landmarks* are similar, but a higher number were analyzed in this paper, mainly at the base of the wing and M vein, based on previous successful results [28, 42]. In any case, the landmark analyses performed by Szpila *et al.* [36] and Grzywacz *et al.* [37] could be standardized to automate GM as a taxonomic tool for Diptera of forensic importance, because they are homologous in other necrophagous families. For comparisons at the intraspecific level, more *landmarks* should be selected [38]. Therefore, depending on the aim of the studies, the landmarks should be carefully chosen.

Geometric morphometrics have been useful to add representative models to the taxonomic groups of insects of medical importance [58-59], discriminating species according to wing configuration. By comparing the centroid size and measure 2, it was revealed that *C. vomitoria* had the greatest wing size, followed by *C. vicina-L. caesar-Ch. megacephala,* then *Ch. albiceps,* while *L. sericata* had the smallest. In addition, each species presented differences between males and females, except *Ch. albiceps* and *Ch. megacephala,* resulting in that females are greater in size than males. In the case of traditional morphometrics, not all the species could be separated according to the lengths measured in this study, although this was possible for *Calliphora* from the Iberian Peninsula and the more common species of *Lucilia*, *L. sericata* and *L. caesar*. For this reason, although the more confident measurement (Measure 2) was used and some taxa could be discriminated, traditional morphometry must be aided by geometric morphometrics to find significant differences among species and between sexes.

Regarding sexual size dimorphism (SSD), a difference in body size between males and females is a widespread phenomenon in plants and animals. The vast

majority of insect species exhibit female-biased SSD [60-61]. Fertility selection is believed to drive the female-biased SSD found in most insect orders. Larger females generally have higher fecundity and often produce larger offspring. Different authors have stated that the ultimate cause of sexual size dimorphism is divergent selection pressure on the sexes [62-63]. In blowflies, females, in addition to feeding on nectar, require a considerable amount of protein to develop viable eggs in their ovaries, so they must look for carrion to obtain proteins and deposit their eggs on it. Since the presence of carrion is somewhat random, females may have advantages to find and colonize it, so they would need more powerful to fly, in addition to having a more developed olfactory capacity than males [64]. Furthermore, in general terms, this hypothesis seems to support the fact that Calliphoridae have a more static region at the wing base (lm 1, lm 12 to lm 17), the rest of the wing forms a more variable region. Similar results have been obtained in Culicidae, where females showed greater variability of shapes than males at the margen and medium area of the wing (the wings of the females were wider in the anterior-posterior sense, while the males had narrower wings); it is indicating a greater sensitivity to the different rearing temperature conditions in females [65-66]. Basal area is involved in the fixation of flight muscles, which are anchored to the thorax because this part of the wings remains constant [67], so females could require a larger wing base attachment because of their larger body size. On the other hand, the fact that there is a variable region could be due to the biological differences present in each of the different species and, therefore, it may be adaptive or simply a specific variation of each species. Phenotypic plasticity in body size is induced by a series of ecological and environmental variables, of which diet quality and developmental temperature are the two most important; the sexes commonly respond differently to environmental variation, and those interactions between environmental variables have different effects on the sexes, affecting patterns of SSD [60].

Brown was one of the first researchers to perform GM studies on Diptera wings, although he also used other distance measurements between wing characters, separating taxa according to wing size and shape [68]. In this study, the separation of taxa into family groups was basically due to shape, rather than to size components, and for each of the calliphorid species, there appeared to be no pattern to the variability between populations, unlike the head/frons ratio used to establish the latitudinal cline in other species of Muscoidea. Brown indicated that wing measurements could be highly

correlated, such that only a limited part of the phenotype was being examined. Other studies [19] confirmed that *L. sericata* females were larger than the males at low density, although, at high densities, the size difference practically disappeared, using TM in wings. Hence, females appeared to experience a greater density-mediated size reduction than males. Moreover, TM in wing has been allowed differentiate populations and hibrids of this species [21]. For this reason, it was interesting in the present study to perform wing size measurements using traditional morphometry, to be able to observe whether differences derived from isometric centroid size corresponded to the differences observed using traditional morphometry. It is easy to think that any length measurement taken is correlated with any other. The combination of both methods would be interesting, since wing size has shown a correlation with body size; the increasing body size in arthropods has been correlated with increasing latitude, suggested that increased body size enhances fecundity and that increased fecundity is beneficial in high latitudes because of relatively short reproductive seasons [67]. Therefore, we could say that Calliphoridae wings do not undergo any allometric process in their development, possibly because the wing has a specific aerodynamic shape compared to the body shape, so that as it increases in size it does not negatively influence the flight. Consequently, it could be said that the wing shape remains stable, regardless of its size [36].

Regarding interspecific differences, when using wing size (measures and centroids), significant differences were found among all of them, except among *C. vicina – L. caesar – Ch. megacephala*. According to some studies, the size and shape of the wings may be related to environmental characteristics, such as temperature [69-70], although, according to other authors, interspecific changes in size and shape have a closer relationship with genotypic variation, considering that there is a genetic basis for explaining these characteristics [71-72]. Considering several studies [73-75], the difference in wing variation found in the present samples could be due to interspecific competition, larval dispersal, and substrate colonization. Growth patterns can vary widely, both among and within species, even when environments are similar [76]. Because body size is an important determinant of reproductive success in many systems, it becomes important to understand the sources of size variation and their consequences. For many organisms, plasticity in adult size and development time, and therefore potentially in SSD, is likely to be an adaptive response to optimize the costs

and benefits of adult size [62]. Species that could show a larger centroid size (a greater possibility of wing variation) could better adapt to different scenarios and, therefore, compete with other species present in the trophic resource. Fertility selection in females probably explains female-biased SSD in most invertebrates, since fertility increases strongly with adult weight [63].

Regarding intraspecific differences, it was shown that there was sexual dimorphism in *C. vicina*, *C. vomitoria*, *L. sericata* and *L. caesar*, while in *Ch. albiceps* and *Ch. megacephala* no significant differences were found. The females need to find sources of protein (carrion), where they oviposit and fertilized. This fact can be demonstrated in succession studies on corpses, where a higher proportion of females than males is observed [61, 77-78]. In *Ch. albiceps* and *Ch. megacephala* no such dimorphism was found, which may be due to the fact that the wing size of this genus is actually related to environmental conditions, as stated by Reigada & Godoy [79], who related the wing and tibiae size in *Ch. megacephala* to monthly temperatures. In their study they obtained a negative correlation between the monthly temperature and the sizes of the two structures. Therefore, the wing size of this genus seems to be more related to temperature since there is no sexual dimorphism, as both sexes experience the same environmental conditions. This fact does not apply to all species, since Giao & Godoy [80] carried out similar studies on *Lucilia eximia* (Wiedermann, 1819), and found that there was no correlation between temperature and wing size. This makes us think that the genus *Chrysomya* does not present sexual dimorphism because the external factors may affect both sexes equally, while the other four species studied show dimorphism, since size may be due to genetic factors that could have settled, producing the differences between males and females. Much of this variation is genetically based and probably due to variation in selection, primarily sexual selection, among species/populations. However, the nutritional effects on physiological correlates of sexual dimorphism (for example, a difference in growth and reproduction needs) are likely to affect the sexes differently, leading to differential plasticity in growth rates and, therefore, in the degree of SSD. Among or within species, SSD occurs through genetic, developmental, and physiological processes that interact with environmental factors such as resource availability, temperature, or mortality risk, producing unique growth paths [75, 81].

Therefore, using techniques related to traditional morphometry, as well as GM, the difference among species becomes evident. These two techniques could be used jointly to explore morphology and identify species [17]. It would be feasible to differentiate one species from another, as well as in some cases to identify the sex of a given individual, based on the wing size and shape, by visual inspection or even automatically. Automatic species identification is not recent [82], in fact there are numerous studies with plants [83], and even with animals, e.g. identification of whales and dolphins considering the edges of their fins [84]. There is another study carried out on animals, specifically on Lepidoptera, by Cosquillo & Romero [85], in which they describe the evaluation of image classification techniques aimed at identifying recognized lepidopteran specimens in Ecuadorian ecological reserves. This basic work of differentiation among fly species of forensic interest is of great importance, specifically in the context of automatic recognition through computer applications, since it allows the development of these techniques. Finally, we would like to point out that these results are preliminary, and we would like to include other species such as flesh-flies and muscids that are forensically important in our region. Data will be entered in databases to obtain a computer application in the future, taking advantage of artificial intelligence [86]. The importance of the recognition of fly species as described here is the fact that wings are structures that are recovered in better conditions from corpses than the rest of the body of the insect, and the identification of these species is essential in forensic sciences in order to use Diptera as evidence in forensic entomology.



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**Table 1.** Mean and standard error of centroid size (different letters indicate significant differences between species; significant sexual dimorphism is indicated with an asterisk; $p<0.05$)

| Species | Centroid size | Centroid size (female) | Centroid size (male) |
|---|---|---|---|
| *Calliphora vicina** | 9.61±1.40[a] | 10.1±1.53[a] | 9.12±1.06[a] |
| *Calliphora vomitoria** | 12.47±1.10[b] | 13.30±0.76[b] | 11.59±0.61[b] |
| *Chrysomya albiceps* | 7.66±0.58[c] | 7.62±0.71[c] | 7.71±0.42[c] |
| *Chrysomya megacephala* | 9.22±0.64[a] | 9.27±0.72[a] | 9.17±0.56[a] |
| *Lucilia caesar** | 9.33±1.01[a] | 9.93±0.73[a] | 8.74±0.90[a] |
| *Lucilia sericata** | 6.80±0.88[d] | 7.12±0.93[d] | 6.47±0.69[d] |

**Table 2.** Mean and standard error of Measure 2 (mm) (different letters indicate significant differences between species; significant sexual dimorphism is indicated with an asterisk; $p<0.05$)

| Species | Measure 2 | Measure 2 (female) | Measure 2 (male) |
|---|---|---|---|
| *Calliphora vicina** | 6.53±0.44[a] | 6.80±1.03[ab] | 6.25±0.72[a] |
| *Calliphora vomitoria** | 8.53±0.45[b] | 9.09±0.52[c] | 7.92±0.42[b] |
| *Chrysomya albiceps* | 5.24±0.40[c] | 5.21±0.49[d] | 5.27±0.30[c] |
| *Chrysomya megacephala* | 6.25±0.44[a] | 6.30±0.49[a] | 6.20±0.38[a] |
| *Lucilia caesar** | 6.31±0.65[a] | 6.69±0.50[b] | 5.94±0.58[a] |
| *Lucilia sericata** | 4.58±0.59[d] | 4.80±0.61[e] | 4.36±0.47[d] |

**Table 3.** Non-parametric test values (F) of the different species studied according to the shape of their wings (*p<0.0001)

| | Males | | | Females | | |
|---|---|---|---|---|---|---|
| | **F** | **PC1 (%)** | **PC2 (%)** | **F** | **PC1 (%)** | **PC2 (%)** |
| *Ch.albiceps – C.vicina** | 171.29 | 66.571 | 6.388 | 262.38 | 74.754 | 5.835 |
| *Ch.albiceps- C.vomitoria** | 61.32 | 43.864 | 9.370 | 159.79 | 65.263 | 7.318 |
| *Ch.albiceps – L.sericata** | 58.91 | 42.502 | 14.986 | 79.87 | 52.161 | 13.285 |
| *Ch.albiceps – L.caesar** | 79.98 | 48.638 | 7.750 | 182.37 | 67.632 | 7.160 |
| *C.vicina – C.vomitoria** | 83.98 | 51.760 | 10.231 | 68.45 | 47.382 | 11.561 |
| *C.vicina – L.sericata** | 30.79 | 28.328 | 24.114 | 19.93 | 26.317 | 19.352 |
| *C.vicina – L.caesar** | 42.41 | 34.736 | 11.270 | 47.09 | 36.576 | 13.218 |
| *C.vomitoria – L.sericata** | 32.02 | 32.176 | 21.276 | 34 | 33.758 | 20.924 |
| *C.vomitoria – L.caesar** | 44.69 | 36.601 | 11.863 | 81.17 | 49.977 | 8.590 |
| *L.sericata – L.caesar** | 9.86 | 25.614 | 10.750 | 10.40 | 30.865 | 15.942 |
| *Ch.megacephala- Ch.albiceps** | 16.47 | 22.316 | 11.789 | 16.42 | 22.488 | 15.429 |
| *Ch.megacephala – C.vicina** | 228.04 | 77.737 | 4.426 | 308.32 | 77.879 | 4.291 |
| *Ch.megacephala – C. vomitoria** | 126.84 | 60.640 | 7.496 | 189.93 | 68.838 | 5.036 |
| *Ch. megacephala – L.sericata** | 107.17 | 55.076 | 13.832 | 89.73 | 54.478 | 12.359 |
| *Ch.megacephala – L.caesar** | 148.83 | 62.645 | 6.928 | 211.38 | 70.908 | 4.751 |

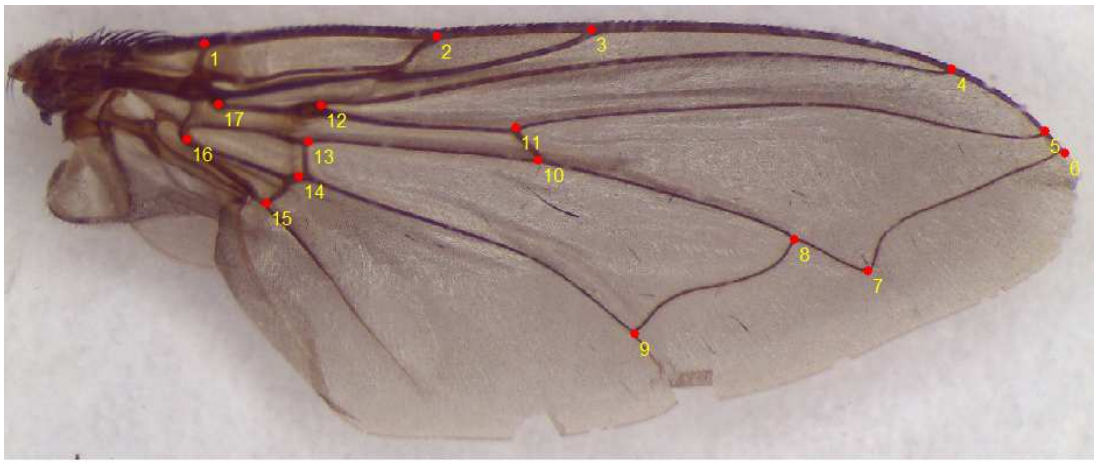



**Fig. 1.** Landmarks on the right wing of *Calliphora vicina*, based on Ludoski et al. 2014 [19]

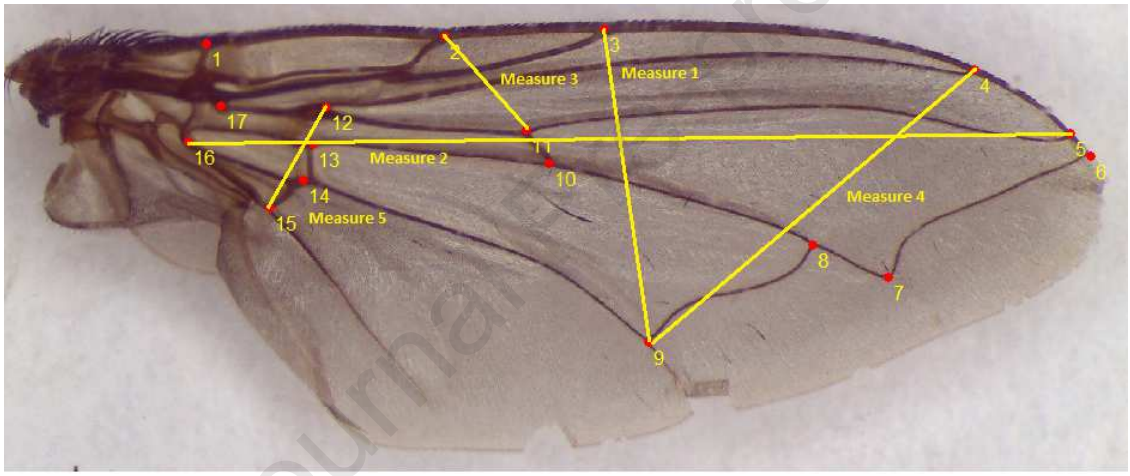



**Fig. 2.** Right wing of *Calliphora vicina* showing the distance between *landmarks* that according to the PCA analysis were more significant (Measures 1-5) in classical morphometrics.

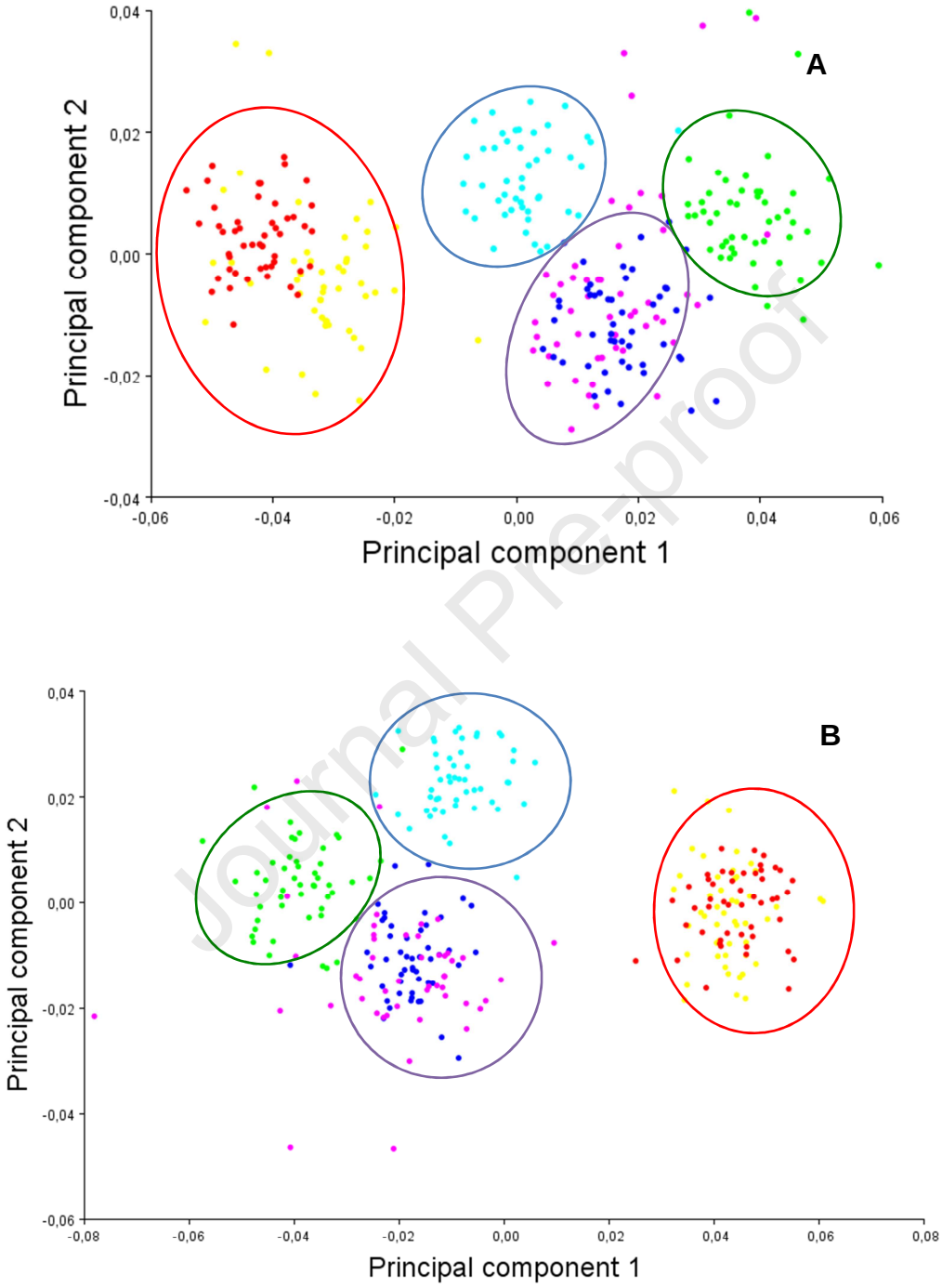



**Fig. 3**. Principal component analysis representation (shape) for males (A) and females (B). In red, *Ch. megacephala*; yellow, *Ch. albiceps*; green, *C. vicina*; light blue, *C. vomitoria*; dark blue, *L. caesar*; and purple, *L. sericata*. The purple line includes *Lucilia* species points, the red line includes *Chrysomya* species points, the green line includes *C. vicina*, and the blue line *C. vomitoria*.

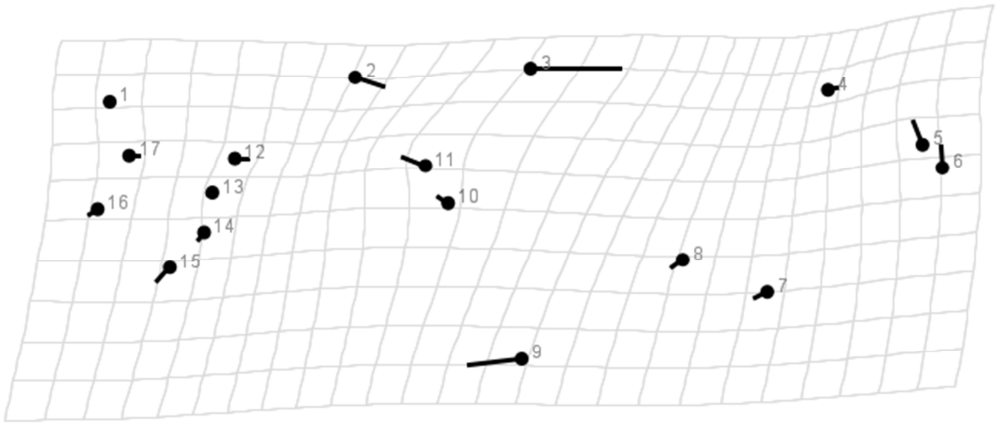



**Fig. 4.** Deformation grid showing differences from average conformation for all blowflies studied

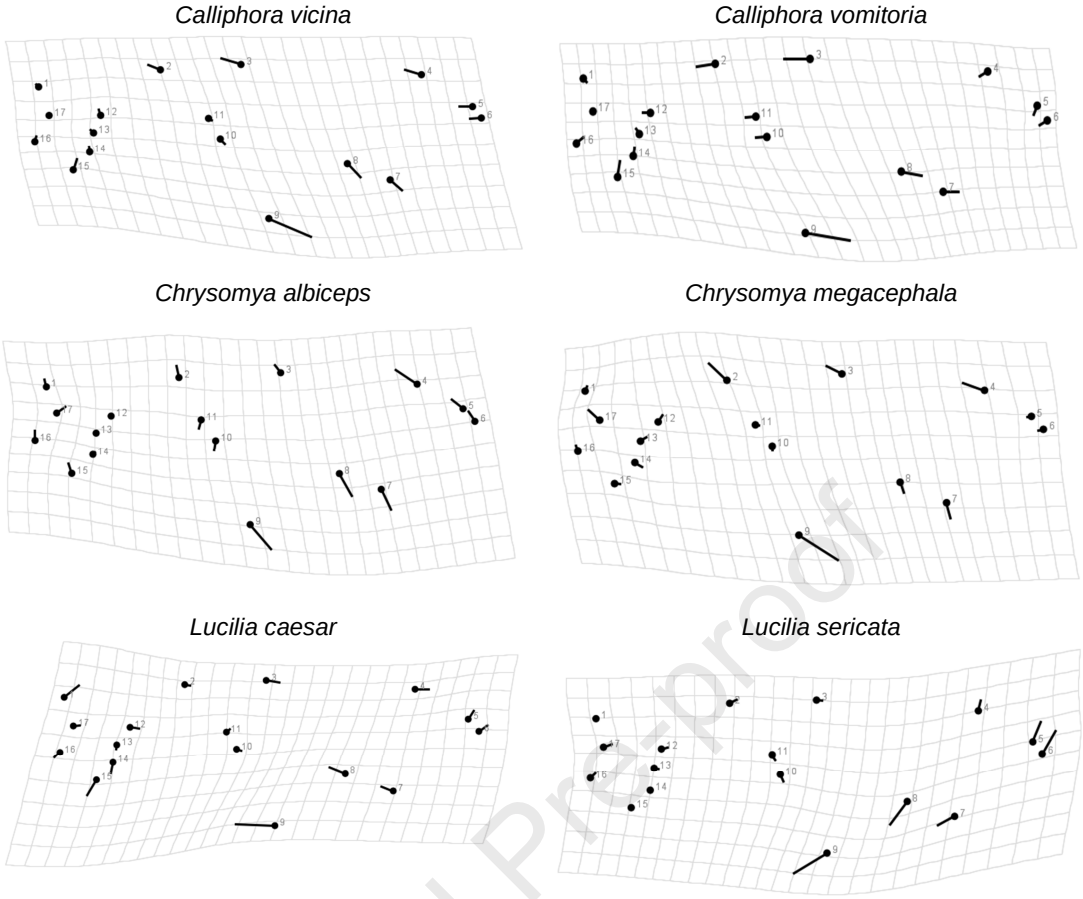



**Fig. 5.** Deformation grids showing differences from the average conformation for each species studied.

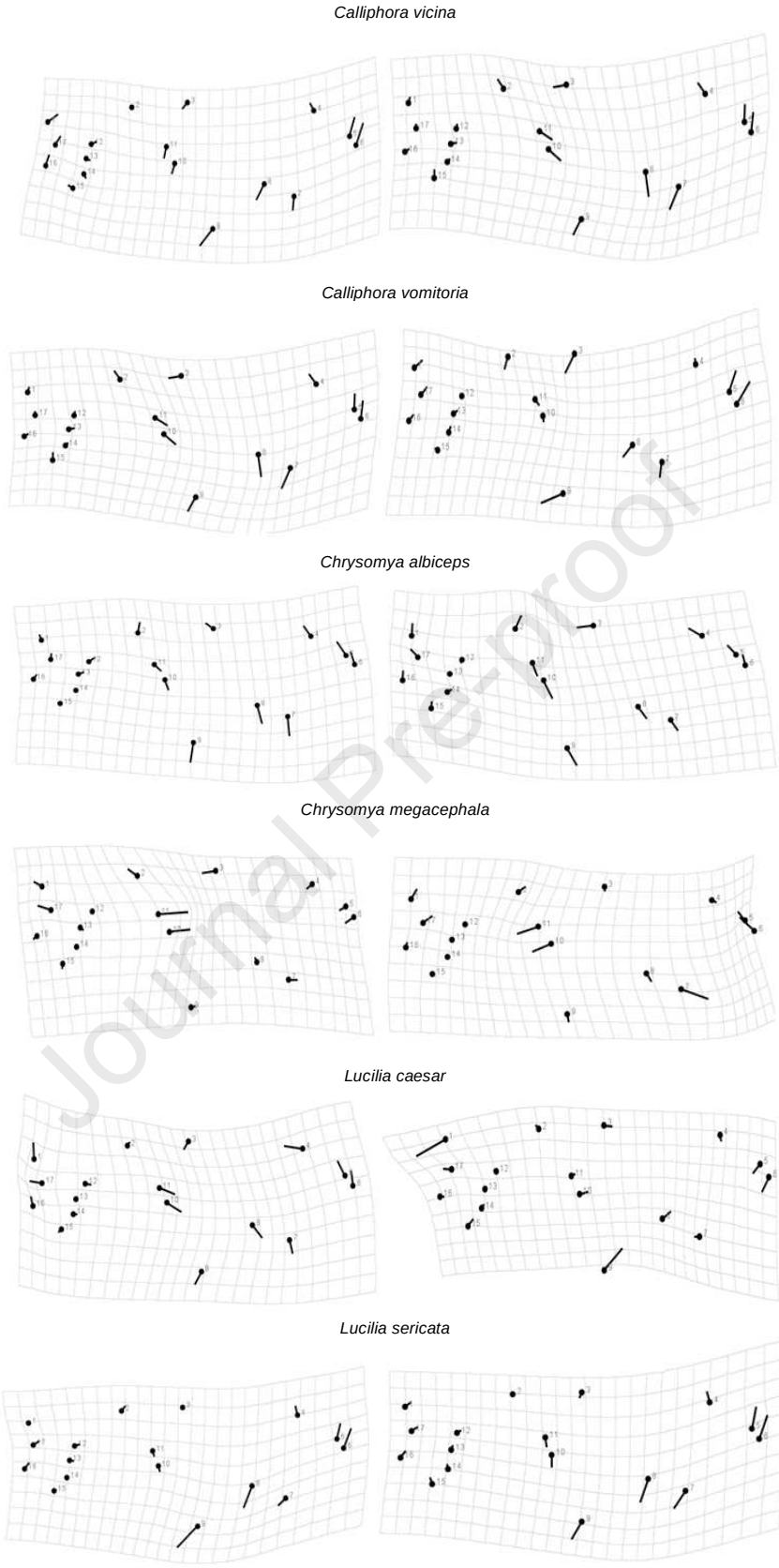
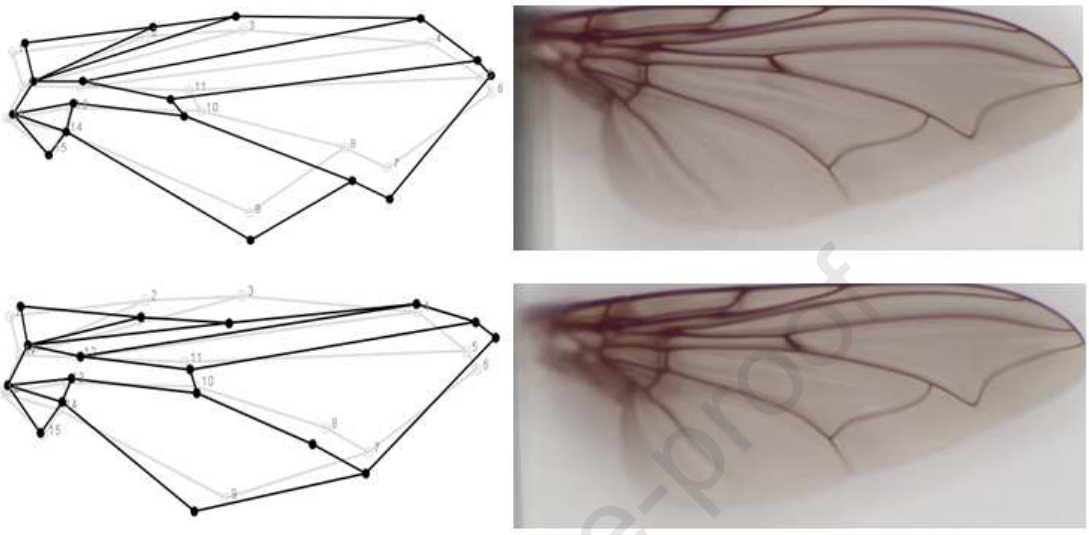


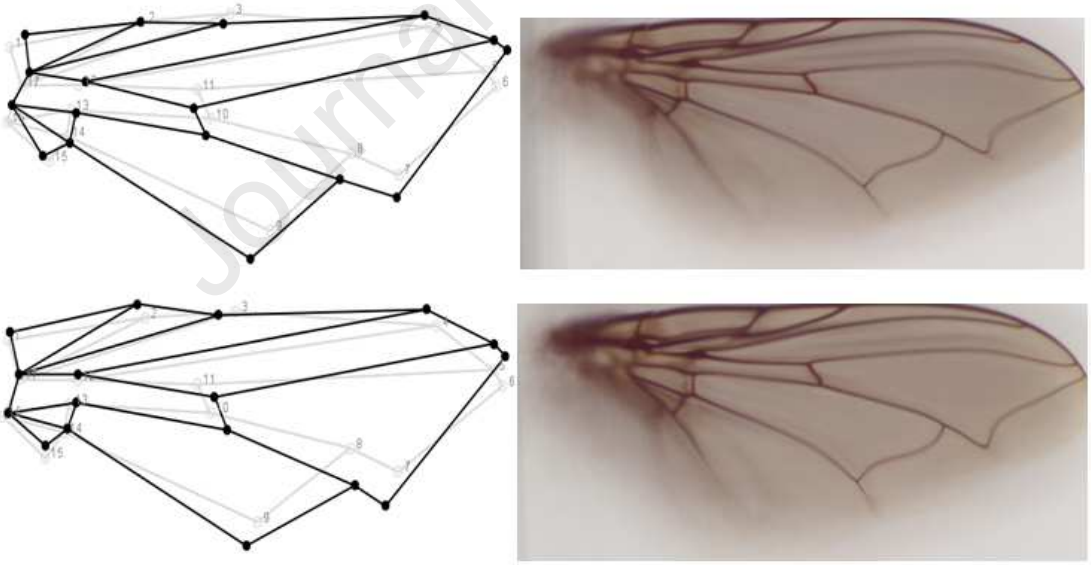

**Fig. 6.** Deformation grids showing differences from the average conformation for each species studied, segregated by sex. On the left, female wings; on the right, male wings.

**ANNEX I:**

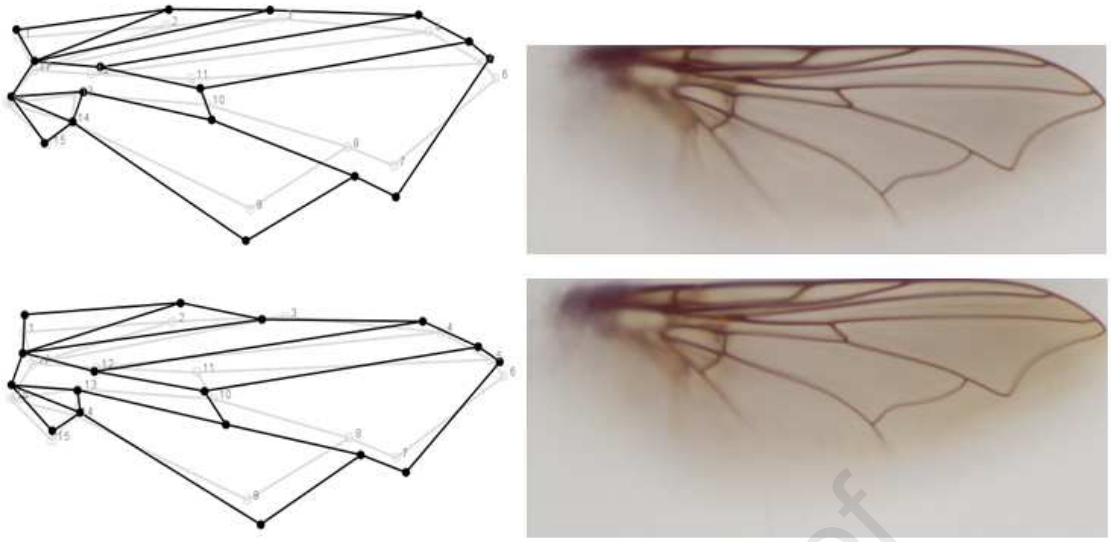
*Calliphora vicina*



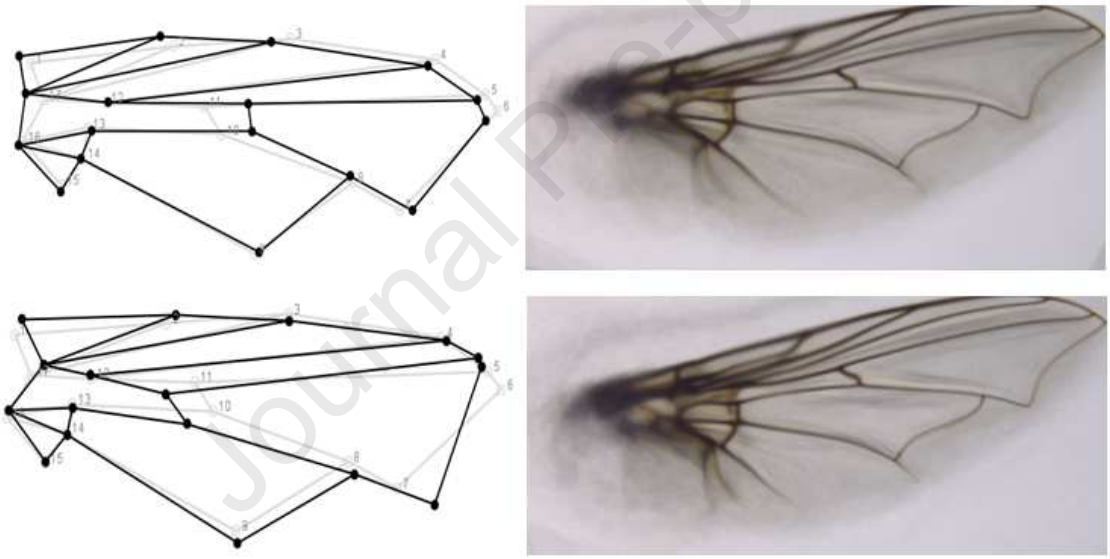
*Calliphora vomitoria*



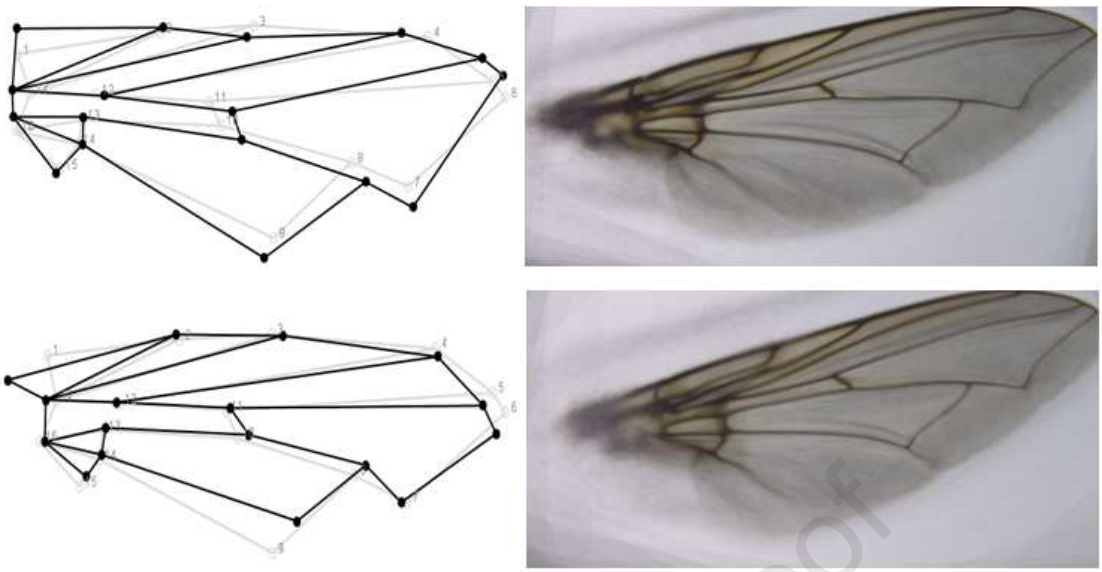
*Chrysomya albiceps*



*Chrysomya megacephala*



*Lucilia caesar*



*Lucilia sericata*

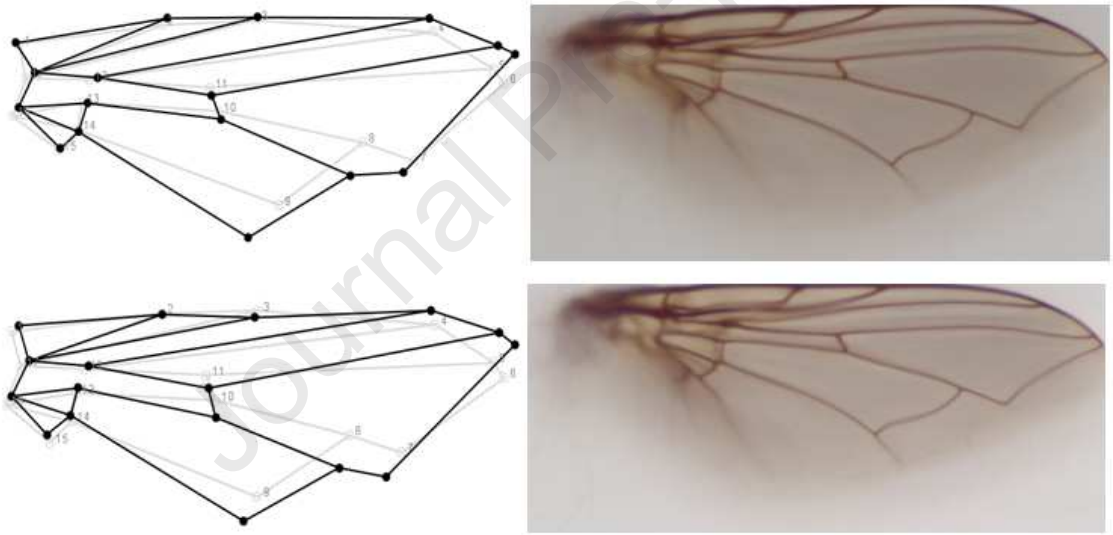


**Fig. 1:** Consensus or "model" wings of the studied species. The upper images of each species correspond to the female, while the lower images correspond to the male. On the left, the variation graphs obtained from the ACP results are shown with a wireframe, where the consensus measure is shown in a dark color and the variance in a lighter one. On the right, we can see the superposition of real images of each wing.

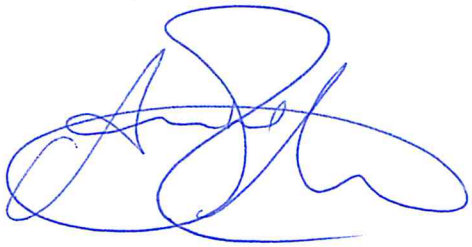
**Highlights:**

- In GM, wing configuration is the best indicator for identifying species of blowflies of forensic importance. Centroid size could be used to differentiate some species, but not all.
- Sexual size dimorphism (SSD) is present in the *Lucilia* and *Calliphora* species, but not in *Chrysomya*. This is important as the females are the most abundant on corpses, because they feed and lay eggs on them.
- Traditional wing morphometrics do not allow the identification of all the commonest forensic blowflies species. Some species have a wide range in size, and they are difficult to distinguish from each other with this technique.
- This study continues with the development of a computer application for the identification of flies that are forensically important, and it will include other families such as Muscidae and Sarcophagidae.

**Conflict of Interest Statement:**

I would like inform to you that there is not conflict of interests regarding the publication of our article titled: **Wing morphometrics for identification of forensically important blowflies (Diptera: Calliphoridae) in Iberian Peninsula**

Sincerely,



Anabel Martínez-Sánchez